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(54) Title: ANTISENSE OLIGONUCLEOTIDES SPECIFIC FOR CDK4

(57) Abstract

Disclosed are oligonucleotides complementary to CDK4 nucleic acids and methods of regulating the G1 to S phase transition in a cell and of inhibiting the growth of a cancer cell using such oligonucleotides. Also disclosed are therapeutic compositions and methods for treating a mammal afflicted with a tumor associated with the aberrant expression of CDK4 and CDK$–associated proteins.

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ANTISENSE OLIGONUCLEOTIDES SPECIFIC FOR CDK4

BACKGROUND OF THE INVENTION

The present invention is related to cell cycle control. More specifically, this invention is directed to oligonucleotides specific for CDK4 nucleic acids which are useful for implementing methods of controlling cell growth and of treating cell cycle disorders such as cancer.

Progression through the cell cycle is controlled at two key points, the transition from G1 to S phase, and from G2 to M phase. The G1-S transition has been termed the restriction point, since it is here that the cell integrates both positive and negative extracellular growth signals and makes the decision to remain quiescent or to begin the process of replication by entering into S phase. A number of proteins have been identified which play a role in the regulation of cell growth by controlling progress through the checkpoints. Of these regulatory proteins, a class of molecules named the cyclin dependent kinases (CDK) have been shown to have a central role in controlling these phase transitions.

The CDK's are serine/threonine kinases which regulate the activity of downstream proteins by phosphorylation. For activity, the CDK's must be complexed with a rate-limiting regulatory subunit termed a cyclin. The cyclins are a family of proteins which are expressed in a cell cycle-dependent manner, with different groups of cyclins being expressed at specific phases of the cell cycle.

The major substrate of the G1 CDK/cyclin complexes is the retinoblastoma (Rb) tumor suppressor protein, which is phosphorylated in a cell cycle dependent manner (DeCaprio et al. (1989) *Cell* 58:1085-1095; Buchkovich et al. (1989) *Cell* 58:1097-1105; Chen et al. (1989) *Cell* 58:1193-1198). In its underphosphorylated state, Rb binds to and inactivates the E2F family of transcription factors (Kaelin et al. (1992) *Cell* 70:351-364; Helin et al. (1992) *Cell* 70:337-350). Upon hyperphosphorylation by G1 CDK/cyclin complexes, Rb releases bound E2F (Hiebert (1992) *Genes Dev.* 6:177-185; Nevins (1992) *Science* 258:424-429), which in turn transactivates a number of genes required for DNA synthesis (DeGregori, J. (1995) *J. Mol. Cell. Biol.* 15:4215-4224), allowing for progression into S phase. The CDK4/cyclin D and CDK6/cyclin D complexes appear to be the primary kinases in the initial phosphorylation of the Rb protein (Weinberg (1995) *Cell* 81:323-330). There are three D-type cyclins (D1, D2, and D3), which are expressed in a cell-type specific manner and which all associate with both CDK4 (Matsushima (1992) *Cell* 71:323-334; Ewen et al. (1993) *Cell* 73:487-497) and CDK6 (Meyerson et al. (1994) *Mol. Cell. Biol.* 14:2077-2086). In addition, the p53 tumor suppressor protein exerts control over the Rb pathway through induction of the CDK inhibitor $p21^{WAF1/CIP1}$ (El-Deiry et al. (1993) *Cell* 75:817-825). A separate family of CDK inhibitors (CDI's) have also been identified which are specific for CDK4 and CDK6, with the p16 protein playing a role in the regulation of RB phosphorylation (Lukas et al. (1995) *Nature* 375:503-510). The normal function of the p16 protein is thought to be to protect the Rb protein from inappropriate

phosphorylation by the CDK4 and CDK6 complexes. In addition to regulation by complex formation with cyclins or interaction with CDI's, CDK activity is also modulated by its phosphorylation state, with activation via phosphorylation on a single threonine residue by the CDK-activating kinase.

The loss of checkpoint control of the cell cycle is a recurring theme in tumorigenesis, with numerous aberrations of the Rb pathway having been identified and associated with specific tumor types. The p16 CDK inhibitor was independently identified as the MTS1 tumor suppressor gene (Kamb et al. (1994) *Nature Genet.* 8:22-26) and has been shown to be the major familial melanoma locus (Kamb et al. (1994) *Nature Genet.* 8:22-26). Inactivation of p16 function has also been associated with a number of tumor types including gliomas (Kyritsis et al. (1996) *Oncogene* 12:63-67; Srivenugopal et al. (1996) *Oncogene* 12:2029-2034), pancreatic adenocarcinomas (Caldas et al. (1994) *Nature Genet.* 8:27-32), pituitary tumors (Woloschak et al. (1996) *Cancer Res.* 56:2493-2496), and bladder cancer (Gonzalez-Zulueta et al. (1995) *Cancer Res.* 55:4531-4535).

The CDK4 gene has been found to be amplified in a number of gliomas (He et al. (1994) *Cancer Res.* 54:5804-5807) and sarcomas (Khatib et al. (1993) *Cancer Res.* 53:5535-5541), and has also been demonstrated to be overexpressed without amplification in a large percentage of metastatic malignant melanomas (Maelandsmo et al. (1996) *British J. Cancer* 73:909-916). More recently, CDK4 was identified as a second familial melanoma locus on the basis of a mutation within the p16

binding domain of CDK4, which prevents binding of p16 and results in the loss of control of CDK4 activity (Zuo et al. (1996) *Nature Genet.* 12:97-99).

The cyclin D1 gene was originally identified as the proto-oncogene locus Bcl1/PRAD1 (Rosenberg et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:9638-9642). Overexpression of cyclin D1 has been associated with parathyroid adenomas (Motokura et al. 1991), centrocytic lymphomas (Withers et al. (1991) *Mol. Cell. Biol.* 11:4846-4853), breast carcinomas (Lammie et al. (1991) *Cancer Cells* 3:413-420), and esophageal carcinomas (Jiang et al. (1992) *Cancer Res.* 52:2980-2983). In cell culture experiments, cyclin D1 overexpression accelerates G1 phase leading to premature entry into S phase (Quelle et al. (1993) *Genes Dev.* 7:1559-1571). It has been demonstrated that inhibition of cyclin D1 function by antisense oligonucleotides (Hung et al. (1996) *Biochem. Biophys. Res. Comm.* 220:719-723), expression of antisense cDNA, or by injection of antibodies directed against the cyclin D1 protein, inhibits cell proliferation by blocking entry into S phase (Hung et al. (1996) *Biochem. Biophys. Res. Comm.* 220:719-723; Baldin et al. (1993) *Genes Dev.* 7:812-821).

Tools and methods for controlling the over-or aberrant expression of CDK4 and cyclin D1 are thus needed, as are methods of controlling cell growth and of treating cell cycle disorders.

SUMMARY OF THE INVENTION

It has been discovered that oligonucleotides directed to the CDK4 mRNA can inhibit the expression of the CDK4 protein, and thereby can regulate the cell cycle. This discovery has been exploited to develop the present invention, which in one aspect includes synthetic oligonucleotides complementary to a CDK4 nucleic acid.

As used herein, the term "oligonucleotide" is meant to include polymers of two or more nucleotides or nucleotide analogs connected together via 5' to 3' internucleotide linkages which may include any linkages that are known in the antisense art. Such molecules have a 3' terminus and a 5' terminus. The term "synthetic oligonucleotide" refers to oligonucleotides synthesized by other than natural processes, such as by biochemical or genetic engineering methods.

As used herein, the term "a CDK4 nucleic acid" refers to genomic DNA and transcript thereof, cDNA, mRNA, and pre-mRNA which encode the CDK4 protein, or portions thereof, 3' or 5' untranslated regions or other regulatory regions, introns, or splice junction sites. The term "complementary to" refers herein to oligonucleotides which are capable of hybridizing or otherwise

associating with at least a portion of such a CDK4 nucleic acid under physiological conditions.

In some embodiments, synthetic oligonucleotides of the invention are complementary to a portion of CDK4 nucleic acid which encodes the 5' untranslated region, the 3' untranslated region, the translational start site, the translational stop site, or a splice junction site. As used herein, the term "splice junction site" is meant to encompass the splice donor, splice acceptor, or intron-exon boundary regions of the nucleic acid.

In some preferred embodiments, the synthetic oligonucleotides of the invention consist essentially of nucleic acids sequences having SEQ ID NO:1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, or 34. Some oligonucleotides of the invention have from about 6 to about 50 nucleic acids. Other oligonucleotides of the invention have from about 15 to about 30 nucleic acids. Yet other oligonucleotide have from 20 to 25 nucleic acids.

In some embodiments of the invention, the oligonucleotides administered are modified with other than phosphodiester-internucleotide linkages between the 5' end of one nucleotide and the 3' end of another nucleotide, in which the 5' nucleotide phosphate has been replaced with any number of chemical groups.

As used herein, the term "modified oligonucleotide" encompasses oligonucleotides with at least one non-phosphodiester internucleotide linkage, modified nucleic acid(s), base(s), and/or sugar(s) other than those found in nature. For example, a 3', 5'-substituted oligonucleotide is an oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position).

A modified oligonucleotide may also be one with added substituents such as diamines, cholestryl, cholesterol, or other lipophilic groups, or a capped species. In addition, unoxidized or partially oxidized oligonucleotides having a substitution in one nonbridging oxygen per nucleotide in the molecule are also considered to be modified oligonucleotides. Also considered as modified oligonucleotides are oligonucleotides having nuclease resistance-conferring bulky substituents at their 3' and/or 5' end(s) and/or various other structural modifications not found in vivo without human intervention are also considered herein as modified.

In some embodiments of the invention, the oligonucleotide has at least one modified internucleotide linkage such as an alkylphosphonate, phosphorothioate, phosphorodithioate, alkylphosphonothioate, alkylphosphonate, phosphoramidate, phosphate ester, carbamate, acetamidate, carboxymethyl ester, carbonate, or phosphate triesters. In some preferred embodiments, the oligonucleotide has phosphorothioate internucleotide linkages.

Oligonucleotides of the invention may include one ribonucleotide which is a 2'-O-substituted ribonucleotide. For purposes of the invention, the term "2'-substituted oligonucleotide" refers to an oligonucleotide having a sugar attached to a chemical group other that a hydroxyl group at its 2' position. The 2'-OH of the ribose molecule can be substituted with -O-lower alkyl containing 1-6 carbon atoms, aryl or substituted aryl or allyl having 2-6 carbon atoms, e.g., 2'-O-allyl, 2'-O-aryl, 2'-O-alkyl (such as a 2'-O-methyl), 2'-halo, or 2'-amino, but not with 2'-H, wherein allyl, aryl, or alkyl groups may be unsubstituted or substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl or amino groups. In some embodiments, the ribonucleotide is a 2'-O-alkylated ribonucleotide such as a 2'-O-methylated ribonucleotide.

Other modified oligonucleotides of the invention have at least one deoxyribonucleotide and at least one ribonucleotide. As used herein, such modified oligonucleotides are termed "chimeric" oligonucleotides. In some embodiments, the chimeric oligonucleotide has at least one 3'-terminal 2'-O-methylated ribonucleotide and/or at least one 5'-terminal 2'-O-methylated ribonucleotide. In some embodiments, all but four or five nucleotides at the 5' terminus of an oligonucleotide of the invention are 2'-substituted ribonucleotides, and in some embodiments, these four or five unsubstituted 5' nucleotides are deoxyribonucleotides. In other embodiments, the oligonucleotide has at least one 2'-substituted ribonucleotide at both its 3' and 5' termini, and in yet other embodiments, the oligonucleotide is

composed of 2'-substituted ribonucleotides in all positions with the exception of at least four or five contiguous deoxyribonucleotide nucleotides in any interior position. In yet other embodiments, chimeric oligonucleotides of the invention have at least two 5'-terminal 2'-O-methylated ribonucleotides and/or at least two 3'-terminal 2'-O-methylated ribonucleotides. In preferred embodiments, the chimeric oligonucleotides of the invention further have modified internucleotide linkages, such as phosphorothioate internucleotide linkages.

In another aspect, the present invention provides a method of regulating the G1 to S phase transition in a cell, comprising the step of administering to the cell an amount of at least one oligonucleotide of the invention sufficient to control the transition. As used herein, the term "G1 to S phase transition" refers to the change the cell goes through between its first growth phase (G1) and its synthesis phase (S) of the cell cycle. A checkpoint normally exists at this transition which controls the rate of cell growth.

The invention also provides a method of regulating the growth of a cell which has lost its G1 to S restriction point control, comprising the step of administering to the cell an amount of an oligonucleotide of the invention sufficient to regain control of the restriction point.

Another aspect of the invention is a method of inhibiting the growth of a cancerous cell which has lost its G1 to S restriction point control, comprising the step of administering to

the cell an amount of an oligonucleotide of the invention sufficient to slow or stop growth of the cell.

Yet another aspect is a therapeutic composition comprising at least one CDK4-specific oligonucleotide of the invention and a pharmaceutically acceptable carrier or diluent. As used herein, a "pharmaceutically or physiologically acceptable carrier or diluent" includes any and all solvents (including but not limited to lactose), dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like.

Also provided by the present invention are methods of treating a cell cycle-related disorder in a mammal and of treating a mammal afflicted with a tumor associated with the aberrant expression of CDK4, cyclin D1, or P16. In this method, a therapeutically effective amount of the therapeutic formulation of the invention is administered to the mammal.

For purposes of the invention, the term "aberrant expression" refers to a decrease, increase, lack of the synthesis and/or function of normal or mutated nucleic acid(s) and/or proteins encoded by a gene. The term "mammal" is meant to encompass primates and humans. The term "therapeutically effective amount" refers to the total amount of each active component of the pharmaceutical formulation or method that is sufficient to show a meaningful subject or patient benefit, i.e., healing of disease conditions characterized by the disease being treated, an increase in rate of healing of such conditions, a reduction in the rate of cell or tumor growth, and a reduction in

CDK4 activity via reduced or altered levels of expression of CDK4 and/or cyclin D) or cells which cause or characterize the disease or disorder being treated.

The subject oligonucleotides and methods of the invention also provide a means of examining the function of the CDK4 gene in a cell, or in a control mammal and in a mammal afflicted with a cell cycle-related disease such as, but not limited to, cancer. The cell or mammal is administered the oligonucleotide, and the expression of CDK4 protein and/or proteins which are known to interact with CDK4 is examined. Presently, gene function is often examined by the arduous task of making a "knock out" animal such as a mouse. This task is difficult, time-consuming and cannot be accomplished for genes essential to animal development since the "knock out" would produce a lethal phenotype. The present invention overcomes the shortcomings of this model.

BRIEF DESCRIPTION OF THE DRAWINGS

The foregoing and other objects of the present invention, the various features thereof, as well as the invention itself may be more fully understood from the following description, when read together with the accompanying drawings in which:

FIG. 1 is a diagrammatic representation of the phases of the cell cycle and the role of CDK4 in the retinoblastoma tumor suppressor protein pathway;

FIG. 2A is a schematic representation of the CDK4 genomic DNA delineating the intron-exon boundaries and splice junction acceptor and donor sites;

FIG. 2B is a schematic representation of the CDK4 cDNA, wherein the 5' UTR is underscored, the 3' UTR is bolded, and the splice junction sites are indicated by a ">----<";

FIG. 3 is a schematic representation of the secondary structure of the CDK4 5'-UTR RNA; and

FIG. 4 is a graphic representation of the inhibition of CDK4 expression in U87 cells, using different an antisense oligonucleotide of the invention, HYB103134 (SEQ ID NO:1), or a sense control oligonucleotide, HYB102644 (SEQ ID NO:35); and

FIG. 5 is a reproduction of a Western blot demonstrating the inhibition of CDK4 protein expression in cells treated with different concentrations of a CDK4-specific oligonucleotide of the invention relative to an actin control.

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DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. The issued U.S. patent, allowed patent applications, and articles cited herein are hereby incorporated by reference.

Progression through the cell cycle is controlled at various checkpoints, the G1 to S and G2 to M being two such phase transitions. Cyclin dependent kinases are the key regulators of these checkpoints, control of which is often lost in some cancer cells. CDK4 is one such protein regulator, the expression of which can be controlled with CDK4-specific oligonucleotides of the present invention.

It is known that antisense oligonucleotides, called an "antisense oligonucleotide," can bind to a target single-stranded nucleic acid molecule according to the Watson-Crick or the Hoogsteen rule of base pairing, and in doing so, disrupt the function of the target by one of several mechanisms: by preventing the binding of factors required for normal transcription, splicing, or translation; by triggering the enzymatic destruction of mRNA by RNase H if a contiguous region of deoxyribonucleotides exists in the oligonucleotide, and/or by destroying the target via reactive groups attached directly to the antisense oligonucleotide.

Oligonucleotides of the invention have a nucleotide sequence which is complementary to a CDK4-specific nucleic acid sequence. Such oligonucleotides can be directed to any portion of a CDK4 nucleic acid, as the genomic sequence is known (Zuo et al. (1996) *Nature Genetics* 12:97-99); FIG. 2A) as is the cDNA sequence (Kanks et al. (1987) *DNAs* 84:388-392; FIG. 2B). One preferable region to which CDK4-specific oligonucleotides of the invention are directed is the 5' untranslated region (UTR) which runs from bases 1 to 227. The 5' UTR of CDK4 is believed to be required for the ability of transforming growth factor-ß (TGF-ß)to induce a G1-specific growth arrest in which CDK4 expression is inhibited at the translational level in a p53-dependent manner (Ewen et al. (1995) *Genes Dev.* 9:204-217). The p53 protein may itself bind to this region which is predicted to be two stable hairpin loops at base pairs 105-138 and base pairs 139-161 (FIG. 3).

Other preferred regions to which oligonucleotides of the invention are directed include, but are not limited to, the 3' UTR running from bases 1-1137, the translational start site (bases 228-230), the translational stop site (bases 1137-1139), and splice junction sites These regions are delineated in FIGS. 2A and 2B.

The oligonucleotides of the invention are at least 6 nucleotides in length, but are preferably 6 to 50 nucleotides long, with 15 to 30mers being common, and 20mers to 25mers being the most common.

Oligonucleotides directed to the translational start or stop site include the three nucleotides complementary to the start or stop site codon and at least 3, preferably at least 7, and more preferably, at least 17 additional nucleotides. Oligonucleotides directed to the splice junction sites are complementary to at least three nucleotides of the splice donor, splice acceptor, or intron/exon boundary.

TABLE 1 lists some nonlimiting representative species of oligonucleotides which are useful in the method of the invention.

TABLE 1

CDK4 Antisense Oligonucleotides

				SEQ. ID.
Oligo	Target	Region	Sequence	NO:
HYB102133	135-154	5' UTR	ggttcctacggccccataca	1
HYB102134	140-159	5' UTR	gagccggttcctacggcccc	2
HYB102925	135-159	5' UTR	gagccggttcctacggcccc ataca	3
HYB102933	131-150	5' UTR	cctacggccccatacacccg	4
HYB102135	112-131	5' UTR	gagctcggtccggagcagct	5
HYB102136	109-128	5' UTR	ctcggtccggagcagctgga	6
HYB102565	211-230	Tr. Start	cattctcagatcaagggaga	7
HYB102566	217-236	Tr. Start	ggtagccattctcagatcaa	8
HYB102567	226-245	Tr. Start	atatcgagaggtagccattc	9
HYB102568	206-225	5' UTR	tcagatcaagggagaccctc	10
HYB102569	1130-1149	Tr. Stop/3' UTR	actccattgctcactccgga	11
HYB102570	1120-1139	Tr. Stop	tcactccggattaccttcat	12
HYB102571	1137-1155	Tr. Stop	cactcgttacctcaccgacg	13
HYB102643	142-161	5' UTR	cggagccggttcctacggcc	14

HYB102645	145-164	5' UTR	ccccggagccggttcctacg	15
HYB102647	140-164	5' UTR	ccccggagccggttcctacg gcccc	16
HYB102925	135-159	5' UTR	gagccggttcctacggcccc ataca	17
HYB102926	1-20	5' UTR	gcgcggaaactgggagggct	18
HYB102927	11-30	5' UTR	ccaaagacgcgcgcggaaac	19
HYB102928	31-50	5' UTR	cctcaccatgtgaccagctg	20
HYB102929	51-70	5' UTR	agaggcccccctcacccccac	21
HYB102930	71-90	5' UTR	agacacaggccgcaagctag	22
HYB102931	91-110	5' UTR	gacgcagagggcccgaccat	23
HYB102932	111-130	5' UTR	agctcggtccggagcagctg	24
HYB102934	151-170	5' UTR	tcggggccccggagccggtt	25
HYB102935	171-190	5' UTR	gctgtgggggcggcccgtta	26
HYB102936	191-210	5' UTR	cctcacgccagcccggggt	27
HYB103170	208-227	5' UTR/SJ	tctcagatcaagggagaccc	28
HYB103171	436-465	SJ	tggcacagacgtccatcagc	29
HYB103172	581-600	SJ	gaaactggcgcatcagatcc	30
HYB103173	749-768	SJ	ggtaccagagtgtaacaacc	31
HYB103174	860-879	SJ	agtttccacagaagaggagc	32
HYB103175	911-930	SJ	ctggaggcagcccaatcagg	33
HYB103176	1047-1066	SJ	gggttaaaagtcagcatttc	34

5' UTR - 5'-untransalted region
3' UTR - 3'-untranslated region
SJ - splice junction
Tr. Stop - translational stop
Tr. Start - translational start

With the published nucleic acid sequences (see, e.g., FIGS. 2A and 2B) and this disclosure provided, those of skill in the art will be able to identify, without undue experimentation, other antisense nucleic acid sequences that inhibit CDK4 expression.

For example, other sequences targeted specifically to human CDK4 nucleic acid can be selected based on their ability to be cleaved by RNase H.

For purposes of the invention, the term "oligonucleotide sequence that is complementary to a nucleic acid sequence" is intended to mean an oligonucleotide sequence that binds to the target nucleic acid sequence under physiological conditions, e.g., by Watson-Crick base pairing (interaction between oligonucleotide and single-stranded nucleic acid) or by Hoogsteen base pairing (interaction between oligonucleotide and double-stranded nucleic acid) or by any other means including in the case of a oligonucleotide binding to RNA, pseudoknot formation. Such binding (by Watson Crick base pairing) under physiological conditions is measured as a practical matter by observing interference with the function of the nucleic acid sequence.

Thus, because of the properties described above, such oligonucleotides are useful therapeutically because of their ability to control or down-regulate the expression of the CDK4 gene in a mammal, according to the method of the present invention.

They are composed of deoxyribonucleotides, ribonucleotides, or a combination of both, with the 5' end of one nucleotide and the 3' end of another nucleotide being covalently linked by non-phosphodiester internucleotide linkages. Such linkages include alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, alkylphosphonates, phosphoramidates, phosphate esters, carbamates, acetamidate,

carboxymethyl esters, carbonates, and phosphate triesters. Preferably, the oligonucleotides of the invention are linked with at least one phosphorothioate internucleotode linkage. Other preferred oligonucleotides of the invention have at least two different internucleotide linkages within the same molecule. For example, U.S. Patent No. 5,149,797 describes traditional chimeric oligonucleotides having a phosphorothioate core region interposed between methylphosphonate or phosphoramidate flanking regions. U.S. Patent Application Ser. No. 08/516,454, filed on August 9, 1995 discloses "inverted" chimeric oligonucleotides comprising one or more nonionic oligonucleotide region (e.g. alkylphosphonate and/or phosphoramidate and/or phosphotriester internucleoside linkage) flanked by one or more region of oligonucleotide phosphorothioate. Oligonucleotides with these linkages can be prepared according to known methods such as phosphoramidate or H-phosphonate chemistry which can be carried out manually or by an automated synthesizer as described by Brown (*A Brief History of Oligonucleotide Synthesis. Protocols for Oligonucleotides and Analogs, Methods in Molecular Biology* (1994) 20:1-8). (See also, e.g., Sonveaux "Protecting Groups in Oligonucleotides Synthesis" in Agrawal (1994) *Meth. Mol. Biol.* 26:1-72; Uhlmann et al. (1990) *Chem. Rev.* 90:543-583).

The oligonucleotides of the composition may also be modified in a number of other ways without compromising their ability to hybridize to the target nucleic acid. Such modifications include, for example, those which are internal or at the end(s) of the oligonucleotide molecule and include additions to the

molecule of the internucleoside phosphate linkages, such as cholesteryl or diamine compounds with varying numbers of carbon residues between the amino groups and terminal ribose, deoxyribose and phosphate modifications which cleave, or crosslink to the opposite chains or to associated enzymes or other proteins which bind to the viral genome. Examples of such modified oligonucleotides include oligonucleotides with a modified base and/or sugar such as arabinose instead of ribose, or a 3', 5'-substituted oligonucleotide having a sugar which, at both its 3' and 5' positions is attached to a chemical group other than a hydroxyl group (at its 3' position) and other than a phosphate group (at its 5' position). Other modified oligonucleotides are capped with a nuclease resistance-conferring bulky substituent at their 3' and/or 5' end(s), or have a substitution in one nonbridging oxygen per nucleotide. Such modifications can be at some or all of the internucleoside linkages, as well as at either or both ends of the oligonucleotide and/or in the interior of the molecule. For the preparation of such modified oligonucleotides, see, e.g., Agrawal (1994) Methods in Molecular Biology 26; Uhlmann et al. (1990) Chem. Rev. 90:543-583).

Oligonucleotides which are self-stabilized are also considered to be modified oligonucleotides useful in the methods of the invention (Tang et al. (1993) Nucleic Acids Res. 20:2729-2735). These oligonucleotides comprise two regions: a target hybridizing region; and a self-complementary region having an oligonucleotide sequence complementary to a nucleic acid sequence that is within the self-stabilized oligonucleotide.

The preparation of these unmodified and modified oligonucleotides is well known in the art (reviewed in Agrawal et al. (1992) Trends Biotechnol. 10:152-158) (see, e.g., Uhlmann et al. (1990) Chem. Rev. 90:543-584; and (1987) Tetrahedron. Lett. 28:(31):3539-3542); Agrawal (1994) Methods in Molecular Biology 20:63-80); and Zhang et al. (1996) J. Pharmacol. Expt. Thera. 278:1-5.

The oligonucleotides administered to the animal may be hybrid oligonucleotides in that they contain both deoxyribonucleotides and at least one 2' substituted ribonucleotide. For purposes of the invention, the term "2'-substituted" means substitution at the 2' position of the ribose with, e.g., a -O-lower alkyl containing 1-6 carbon atoms, aryl or substituted aryl or allyl having 2-6 carbon atoms e.g., 2'-O-allyl, 2'-O-aryl, 2'-O-alkyl, 2'-halo, or 2'-amino, but not with 2'-H, wherein allyl, aryl, or alkyl groups may be unsubstituted or substituted, e.g., with halo, hydroxy, trifluoromethyl, cyano, nitro, acyl, acyloxy, alkoxy, carboxyl, carbalkoxyl or amino groups. Useful substituted ribonucleotides are 2'-0-alkyls such as 2'-0-methyl.

The hybrid DNA/RNA oligonucleotides useful in the method of the invention resist nucleolytic degradation, form stable duplexes with RNA or DNA, and preferably activate RNase H when hybridized with RNA. They may additionally include at least one unsubstituted ribonucleotide. For example, an oligonucleotide of the invention may contain all deoxyribonucleotides with the exception of at least one or at least

two 2'-substituted ribonucleotides at the 3'-terminus or the 5'-terminus of the oligonucleotide. Alternatively, the oligonucleotide may have at least one or at least two substituted ribonucleotide at both its 3' and 5' termini.

One preferred class of oligonucleotides of the invention contains four or more deoxyribonucleotides in a contiguous block, so as to provide an activating segment for RNase H. In certain cases, more than one such activating segment will be present at any location within the oligonucleotide. There may be a majority of deoxyribonucleotides in oligonucleotides according to the invention. In fact, such oligonucleotides may have as many as all but one nucleotide being deoxyribonucleotides. Thus, a preferred oligonucleotide having from about 6 to about 50 nucleotides or most preferably from about 20 to about 25 nucleotides, the number of deoxyribonucleotides present ranges from 1 to about 24. Other useful oligonucleotides may consist only of 2'-substituted ribonucleotides. Some non-limiting representative hybrid oligonucleotides of the invention are shown in Table 2 below, where the 2'-substituted ribonucleotides are underscored.

TABLE 2

Hybrid CDK4 Antisense Oligonucleotides

Oligo	Target	Sequence	SEQ ID NO:
HYB102133	135-154	ggttcctacggccccataca	1
HYB102133	135-154	ggttcctacggccccataca	1
HYB102133	135-154	ggttcctacggccccataca	1
HYB102133	135-154	ggttcctacggccccataca	1
HYB102133	135-154	gguucctacggccccauaca	36
HYB102133	135-154	ggutcctacggccccataca	37
HYB102133	135-154	ggttcctacggccccauaca	1
HYB102133	135-154	gguucctacggccccataca	38
HYB102133	135-154	ggutcctacggccccataca	39
HYB102133	135-154	ggttcctacggccccataca	1
HYB102133	135-154	gguuccuacggccccauaca	40
HYB102134	140-159	gagccggttcctacggcccc	2
HYB102134	140-159	gagccggttcctacggcccc	2
HYB102134	140-159	gagccggttcctacggcccc	2
HYB102134	140-159	gagccggttcctacggcccc	2
HYB102134	140-159	gagccggttcctacggcccc	2
HYB102134	140-159	gagccggttcctacggcccc	2
HYB102134	140-159	gagccggttcctacggcccc	2
HYB102134	140-159	gagccggttcctacggcccc	2
HYB102134	140-159	gagccggttcctacggcccc	2
HYB102134	140-159	gagccgguuccuacggcccc	41

The oligonucleotides according to the invention are effective in inhibiting the expression of CDK4, and particularly in inhibiting protein expression, in cells *in vivo* or *in vitro* The ability to inhibit the expression of the CDK4 protein is clearly important to the treatment of a variety of cell cycle related disorders.

One aspect of the invention provides therapeutic compositions suitable for treating disorders resulting from the loss of cell cycle control at a checkpoint, such as uncontrolled cell growth resulting in cancer or tumorigenesis. Such a therapeutic composition includes at least one CDK4-specific oligonucleotide of the invention and a pharmaceutically or physiologically acceptable carrier of diluent. As used herein, a "pharmaceutically or physiologically acceptable carrier or diluent" includes any and all solvents (including but not limited to lactose), dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use in the therapeutic compositions of the invention is contemplated. Supplementary active ingredients can also be incorporated into the compositions.

In one preferred therapeutic composition of the invention, about 25 to 75 mg of a lyophilized oligonucleotide having a nucleotide sequence selected from the group consisting of SEQ ID NOS:1-34 and 20-75 mg lactose, USP, which is reconstituted with sterile normal saline to the therapeutically effective dosages described herein. Another preferred therapeutic composition of the invention comprises about 50 mg of an oligonucleotide having SEQ ID NO:1 and about 40 mg lactose.

Another aspect of the invention provides methods for treating mammals suffering from a disease or disorder caused by expression of an aberrant gene or the overexpression of the

CDK4 gene. In this method a therapeutically effective amount of a therapeutic composition of the invention is administered to the mammal. Such methods of treatment according to the invention, may be administered in conjunction with other anticancer therapeutic agents or treatments, e.g., melphalan.

As used herein, the term "therapeutically effective amount" refers to the total amount of each active component of the pharmaceutical formulation or method that is sufficient to show a meaningful subject or patient benefit, i.e., a reduction in or arrest of the growth rate of the tumor or a reduction in the size of the cancer or tumor; healing of disease conditions characterized by the particular disorder being treated and/or an increase in rate of healing of such conditions; and a reduction in the rate of expression of proteins (e.g., CDK4 and/or cyclin D) which directly or indirectly cause or characterize the disease or disorder being treated . When applied to an individual active ingredient, administered alone, the term refers to that ingredient alone. When applied to a combination, the term refers to combined amounts of the active ingredients that result in the therapeutic effect, whether administered in combination, serially or simultaneously.

A "therapeutically effective manner" refers to a route, duration, and frequency of administration of the pharmaceutical formulation which ultimately results in meaningful patient benefit, as described above. In some embodiments of the invention, the pharmaceutical formulation is administered via injection, sublingually, rectally, intradermally, orally, or enterally

in bolus, continuous, intermittent, or continuous, followed by intermittent regimens.

The therapeutically effective amount of synthetic oligonucleotide in the pharmaceutical composition of the present invention will depend upon the nature and severity of the condition being treated, and on the nature of prior treatments which the patent has undergone. Ultimately, the attending physician will decide the amount of synthetic oligonucleotide with which to treat each individual patient. Initially, the attending physician will administer low doses of the synthetic oligonucleotide and observe the patient's response. Larger doses of synthetic oligonucleotide may be administered until the optimal therapeutic effect is obtained for the patient, and at that point the dosage is not increased further. It is contemplated that the dosages of the pharmaceutical compositions administered in the method of the present invention should contain about 10 μg to about 20 mg of synthetic oligonucleotide per kg body or organ weight, preferably 0.1 to 5.0 mg/kg body weight per day, and more preferably 0.1 to 2.0 mg/kg body weight per day. When administered systemically, the therapeutic composition is preferably administered at a sufficient dosage to attain a blood level of oligonucleotide from about 0.01 μM to about 10 μM. Preferably, the concentration of oligonucleotide at the site of aberrant gene expression should be from about 0.01 μM to about 10 μM, and most preferably from about 0.05 μM to about 5 μM. However, for localized administration, much lower concentrations than this may be effective, and much higher concentrations may be tolerated. It may be desirable to

administer simultaneously or sequentially a therapeutically effective amount of one or more of the therapeutic compositions of the invention when individual as a single treatment episode.

Administration of pharmaceutical compositions in accordance with invention or to practice the method of the present invention can be carried out in a variety of conventional ways, such as by oral ingestion, enteral, rectal, or transdermal administration, inhalation, sublingual administration, or cutaneous, subcutaneous, intramuscular, intraocular, intraperitoneal, or intravenous injection, or any other route of administration known in the art for administrating therapeutic agents.

When the composition is to be administered orally, sublingually, or by any non-injectable route, the therapeutic formulation will preferably include a physiologically acceptable carrier, such as an inert diluent or an assimilable edible carrier with which the composition is administered. Suitable formulations that include pharmaceutically acceptable excipients for introducing compounds to the bloodstream by other than injection routes can be found in Remington's Pharmaceutical Sciences (18th ed.) (Genarro, ed. (1990) Mack Publishing Co., Easton, PA). The oligonucleotide and other ingredients may be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the individual's diet. The therapeutic compositions may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. When

the therapeutic composition is administered orally, it may be mixed with other food forms and pharmaceutically acceptable flavor enhancers. When the therapeutic composition is administered enterally, they may be introduced in a solid, semi-solid, suspension, or emulsion form and may be compounded with any number of well-known, pharmaceutically acceptable additives. Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are also contemplated such as those described in U.S. Patent Nos. 4,704,295, 4,556,552, 4,309,404, and 4,309,406.

When a therapeutically effective amount of composition of the invention is administered by injection, the synthetic oligonucleotide will preferably be in the form of a pyrogen-free, parenterally-acceptable, aqueous solution. The preparation of such parenterally-acceptable solutions, having due regard to pH, isotonicity, stability, and the like, is within the skill in the art. A preferred pharmaceutical composition for injection should contain, in addition to the synthetic oligonucleotide, an isotonic vehicle such as Sodium Chloride Injection, Ringer's Injection, Dextrose Injection, Dextrose and Sodium Chloride Injection, Lactated Ringer's Injection, or other vehicle as known in the art. The pharmaceutical composition of the present invention may also contain stabilizers, preservatives, buffers, antioxidants, or other additives known to those of skill in the art.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable

solutions or dispersions. In all cases the form must be sterile. It must be stable under the conditions of manufacture and storage and may be preserved against the contaminating action of microorganisms, such as bacterial and fungi. The carrier can be a solvent or dispersion medium. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents. Prolonged absorption of the injectable therapeutic agents can be brought about by the use of the compositions of agents delaying absorption. Sterile injectable solutions are prepared by incorporating the oligonucleotide in the required amount in the appropriate solvent, followed by filtered sterilization.

The pharmaceutical formulation can be administered in bolus, continuous, or intermittent dosages, or in a combination of continuous and intermittent dosages, as determined by the physician and the degree and/or stage of illness of the patient. The duration of therapy using the pharmaceutical composition of the present invention will vary, depending on the unique characteristics of the oligonucleotide and the particular therapeutic effect to be achieved, the limitations inherent in the art of preparing such a therapeutic formulation for the treatment of humans, the severity of the disease being treated and the condition and potential idiosyncratic response of each individual patient. Ultimately the attending physician will decide on the appropriate duration of intravenous therapy using the pharmaceutical composition of the present invention.

Human diseases and disorders which are caused by expression of an aberrant gene may be treated in accordance with the methods of the invention and have been discussed earlier in this disclosure.

The therapeutic pharmaceutical formulation containing the oligonucleotide includes a physiologically acceptable carrier, such as an inert diluent or an assimilable edible carrier with which the peptide is administered. Suitable formulations that include pharmaceutically acceptable excipients for introducing compounds to the bloodstream by other than injection routes can be found in Remington's Pharmaceutical Sciences (18th ed.) (Genarro, ed. (1990) Mack Publishing Co., Easton, PA). The oligonucleotide and other ingredients may be enclosed in a hard or soft shell gelatin capsule, compressed into tablets, or incorporated directly into the individual's diet. The oligonucleotide may be incorporated with excipients and used in the form of ingestible tablets, buccal tablets, troches, capsules, elixirs, suspensions, syrups, wafers, and the like. When the oligonucleotide is administered orally, it may be mixed with other food forms and pharmaceutically acceptable flavor enhancers. When the oligonucleotide is administered enterally, they may be introduced in a solid, semi-solid, suspension, or emulsion form and may be compounded with any number of well-known, pharmaceutically acceptable additives. Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are also contemplated such as those described in U.S. Patent Nos. 4,704,295, 4,556,552, 4,309,404, and 4,309,406.

It will be appreciated that the unit content of active ingredient or ingredients contained in an individual dose of each dosage form need not in itself constitute an effective amount since the necessary effective amount can be reached by administration of a plurality of dosage units (such as capsules or tablets or combinations thereof).

In order to determine whether antisense oligonucleotides of the invention complementary to CDK4 nucleic acid are able to control CDK4 expression, cells treated with such oligonucleotides were analyzed for CDK4 protein. CDK4 protein levels were examined by Western blot in U-87 human glioglastoma cells following treatment with CDK4-specific oligonucleotide concentrations ranging from 0.1 μ m to 1.0 μ m. As shown in FIG. 4, levels of CDK4 protein were decreased in a concentration-dependent manner following treatment with the HYB102134 (SEQ ID NO:2) oligonucleotide. A maximum inhibition of about 78% was observed with the two anti-CDK4 oligonucleotides of the invention as compared to nonspecific inhibition of 27% by a reverse sequence control oligonucleotide HYB102644 (5'-gggagccggttcctacggcc-3' (SEQ ID NO:42)). Similar results were obtained with anti-CDK4 oligonucleotide HYB102133 (SEQ ID NO:1).

In order to assess the effect of antisense inhibition of CDK4 on progression through the cell cycle, FACS analysis was employed. U-87 cells were quasi-synchronized by serum starvation prior to oligonucleotide treatment. Following treatment, cells were released from the block by the addition of

serum and then harvested after 18 hours of growth. The cells were fixed, stained with propidium iodine, and the DNA content was analyzed by FACS to determine the percentage of cells in each phase of the cell cycle. A decrease in CDK4 activity would be predicted to result in an inhibition of G1 to S progression, and thus an increase in the percentage of cells remaining in G1. Following treatment with HYB102133 and HYB102134, the percentage of U-87 cells in G1 was 51% and 47%, respectively, as compared to 38% of untreated cells. This experiment suggests that antisense inhibition of CDK4 expression may result in at least partial re-establishment of the G1/S checkpoint control lost by the deregulation of endogenous CDK4.

The following examples illustrate the preferred modes of making and practicing the present invention, but are not meant to limit the scope of the invention since alternative methods may be utilized to obtain similar results.

EXAMPLES

1. Synthesis and Analysis of Oligonucleotides

Oligonucleotides of the invention and control oligonucleotides were synthesized, purified, and analyzed as follows.

Phosphorothioate deoxynucleosides were synthesized on CPG on a 5-6 μ mole scale on an automated synthesizer (model 8700, Millipore, Bedford, MA) using the H-phosphonate approach described in U.S. Patent No. 5,149,798. Deoxynucleoside H-phosphonates were obtained from Millipore (Bedford, MA). 2'-O-methyl ribonucleotide H-phosphonates or phosphorothioates were synthesized by standard procedures (see, e.g., "Protocols for Oligonucleotides and Analogs" in Meth. Mol. Biol. (1993) Vol. 20) or commercially obtained (e.g., from Glenn Research, Sterling, VA and Clontech, Palo Alto, CA). Segments of oligonucleotides containing 2'-O-methyl nucleoside(s) were assembled by using 2'-O-methyl ribonucleoside H-phosphonates or phosphorothioates for the desired cycles. Similarly, segments of oligonucleotides containing deoxyribonucleosides were assembled by using deoxynucleoside H-phosphonates for the desired cycles. After assembly, CPG bound oligonucleotide H-phosphonate was oxidized with sulfur to generate the phosphorothioate linkage. Oligonucleotides were then deprotected in concentrated NH_4OH at 40°C for 48 hours.

Crude oligonucleotide (about 500 A_{260} units) was analyzed on reverse low pressure chromatography on a C_{18} reversed phase medium. The DMT group was removed by treatment with 80% aqueous acetic acid, then the oligonucleotides were dialyzed against distilled water and lyophilized.

2. Antisense Oligonucleotide Treatment of Cells

U-87 human glioblastoma cells (ATCC HTB-14, American Type Culture Collection, Rockville, MD) were plated at a density of 2.5 x 10^5 cells per 2 ml in wells of six-well tissue culture plates and incubated overnight. U-87 MG . The cells were maintained in MEM medium, supplemented with 1 mM sodium pyruvate, 100 I.U./ml penicillin, 100 μ g/ml streptomycin, and 10% fetal calf serum. Uptake of oligonucleotide by U-87 cells was facilitated by the use of the transfection reagent Lipofectin (Life Technologies, Gaithersburg, MD). Lipofectin (4 μ g) was added together with various concentrations of oligonucleotide in a total volume of 100 μ l of Optimem transfection media (Life Technologies, Gaithersburg, MD) and incubated at room temperature for 30 min. The U-87 cells were washed with 2 ml of Optimem (Life Technologies, Gaithersburg, MD) and then 0.9 ml of Optimem was added to each well. The 100 μ l mix of lipofectin/oligonucleotides was then added to the cells, which were incubated for 5 hr at 37° C. Following the incubation period, 1 ml of MEM with 20% fetal calf serum was added to each well. The cells were then incubated at 37° C for varying times, depending on the assay to be conducted.

3. Western Analysis

Following oligonucleotide treatment and overnight incubation, the U-87 cells were washed with 2 ml of PBS and then lysed in 200 μl of 3x sample buffer (188 mM Tris, pH 6.8, 6% SDS, 30% glycerol, 1.5% 2-mercaptoethanol, 0.005% bromphenol blue). The cell lysates were boiled for 5 minutes before 50 μl was loaded and electrophoresed on a 10% SDS polyacrylamide gel. Proteins were transferred to Immobilon-P (Millipore, Bedford, MA 01730) membrane using a Trans-Blot SD apparatus (Bio-Rad Laboratories, Hercules, CA 94547) at 12 volts for 45 min. Following transfer, the membrane was rinsed in TTBS (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) and blocked for 1 hr at room temperature in TTBS, 10% nonfat dry milk. The membrane was then incubated for 1 hr at room temperature with a rabbit anti-CDK 4 polyclonal antibody in TTBS, 10% nonfat dry milk, washed once for 15 min, and twice for 5 min in TTBS. Incubation with a anti-rabbit HRP conjugate (Promega, Madison, WI) diluted 1:7500 was for 30 min at room temperature, followed by washes as above. Visualization of CDK4 protein was accomplished by incubation in ECL chemiluminesence reagent (Amersham, Arlington Heights, IL) for 1 min followed by autoradiagraphy. In order to standardize the amount of cell lysate for each sample, the blot was reprobed with an anti-B-actin antibody at 1 μ g/ml (Oncogene Science, Uniondale, NY) using the above procedure. Representative results are shown in FIG. 5.

4. Northern Analysis

Cells were treated with oligonucleotides as described above and incubated at 37° C for 18-24 hr before total RNA was isolated using TRIzol Reagent (Life Technologies, Gaithersburg, MD). The cells were harvested by trypsinization, washed with PBS, and lysed cells in 1 ml of TRIzol Reagent. Following incubated for 5 min at room temperature, 0.2 ml of chloroform was added and the samples were incubated at room temperature for 2 to 3 min. The samples were centrifuged for 15 min at 4° C, and the RNA was then precipitated from the aqueous phase by addition of isopropyl alcohol. The RNA pellet was rinsed with 75% ethanol, briefly air dried, and resuspended in 11 μl of RNase-free dH_2O, to which was added 4 μl of 10x MOPS buffer, 5 μl of formaldehyde, and 20 μl of formamide. The samples were incubation for 15 min at 65° C before electrophoresis through a 1% agarose gel (SeaKem ME, FMC, Rockland, ME) containing 10% formaldehyde. Following electrophoresis, the gel was rinsed in dH_2O for 5 min, and then in 10x SSC for 30 min. The RNA was blot-transferred to Zeta-Probe GT membrane (BioRad) and immobilized by UV crosslinking. A full length Bam H1 CDK4 cDNA fragment was ^{32}P labelled using a Random Primer Kit (RPN 1606, Amersham, Arlington Heights, IL). The template cDNA (25 ng) and primers were mixed in a final volume of 20 μl and denatured by heating to 100°C for 5 minutes. To the denatured template/primer mix was added 10 μl of labeling buffer, 5 μl of ^{32}P dCTP (Amersham, Arlington Heights, IL), and 2 μl of Klenow. The labeling reaction was incubated at 37° C for 10 min and stopped by the addition of 5

μl 0.2 M EDTA. The labeled probe was purified from unincorporated isotope using Quick Spin Columns (Boehringer Mannheim, Indianapolis, IN). The RNA blot was prehybridized at 65° C for 5 min in hybridization solution (0.25 M Na_2HPO_4, 7% SDS, pH 7.2). The purified radiolabelled probe was added to a concentration of 10^6 cpm/ml and incubated overnight at 65° C with agitation. The membrane was then washed 2 times with 2 x SSC/0.1 % SDS for 15 min each at 65° C, and once with 0.2 x SSC/0.1 % SDS for 15 min at 65° C, followed by autoradiography.

5. Assay for Induction of G1-Specific Growth Arrest

In order to assay for induction of a G1-specific block by inhibition of CDK4 expression with anti-sense oligonucleotides, U-87 cells were first quasi-synchronized by serum starvation. Cells were plated at 2.5 x 10^5 per well of a six-well tissue culture plate and incubated overnight at 37°C. The following day, the cells were washed twice with MEM, 0.1% fetal calf serum, before the addition of 2 ml per well of the same media and incubation at 37° C for 48-64 hours. The cells were treated with oligonucleotides as described above and released from the serum block upon the addition of MEM, 20% fetal calf serum. After 18 hr at 37°C, the cells were harvested by trypsinization and washed three times in PBS. The cells were then fixed in 70% ethanol for 1 hr at -20° C. Following two washes with PBS, the cells were resuspended in 1.1% sodium citrate and 2.5 μg of RNase (Boehringer Mannheim, Indianapolis, MD) was added before incubation at 37° C for 20 min. propidium iodine (250 μl of 50

μg/ml stock in PBS, 2% fetal calf serum) was then added, with incubation at 37° C continuing for an additional 20 min. FACS was employed to analyze the cell cycle profile of treated cells verses controls and determine the percentage of the cell populations blocked in G1 phase.

6. Cell Proliferation Assay

U-87 cells (ATCC HTB-14, American Type Culture Collection, Rockville, MD) were plated in 96-well plates to a density of 1 x 10^4 cells per well, and incubated overnight at 37° C. A 10x mixture of Lipofectin (4 μg/ml final concentration) and oligonucleotide (at various concentrations) was made in Optimem (Life Technologies, Gaithersburg, MD) and incubated at room temperature for 30 min. Cells were washed once in Optimem and then 90 μl of same media was added per well. 10 μl of the Lipofectin/oligonucleotide mix was added per well and the cells were incubated for 5 hr at 37° C. 100 μl of MEM, 20% fetal calf serum was added per well and the cells were incubated overnight, at which time the media was changed with MEM, 10% fetal calf serum. The cells were incubated for 24 to 72 hr and cell proliferation was measured using the CellTiter 96 AQ Assay (Promega, Madison, WI). Proliferation is expected to be reduced in the presence of CDK4-specific oligonucleotides of the invention.

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the scope of this invention, and are covered by the following claims.

What is claimed is:

1. A synthetic oligonucleotide complementary to a CDK4 nucleic acid.

2. The synthetic oligonucleotide of claim 1 which is complementary to a portion of CDK4 nucleic acid which encodes the 5' untranslated region, the 3' untranslated region, the translational start site, the translational stop site, or a splice junction site.

3. The synthetic oligonucleotide of claim 1 consisting essentially of a nucleic acid sequence selected from the group consisting of SEQ ID NOS:1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, and 34.

4. The oligonucleotide of claim 1 having 6 to 50 nucleic acids.

5. The oligonucleotide of claim 4 having 15 to 30 nucleic acids.

6. The oligonucleotide of claim 5 having 20 to 25 nucleic acids.

7. The oligonucleotide of claim 1 which is modified.

8. The modified oligonucleotide of claim 7 comprising an internucleotide linkage selected from the group consisting of alkylphosphonates, phosphorothioates, phosphorodithioates, alkylphosphonothioates, phosphoramidates, phosphoramidites, phosphate esters, carbamates, carbonates, phosphate triesters, acetamidate, and carboxymethyl esters.

9. The oligonucleotide of claim 8 comprising at least one phosphorothioate internucleotide linkage.

10. The oligonucleotide of claim 8 comprising phosphorothioate internucleotide linkages.

11. The oligonucleotide of claim 1 comprising at least one deoxyribonucleotide.

12. The oligonucleotide of claim 1 comprising at least one ribonucleotide.

13. The oligonucleotide of claim 12 wherein the ribonucleotide is a 2'-O-substituted ribonucleotide.

14. The oligonucleotide of claim 13 wherein the ribonucleotide is a 2'-O-alkylated ribonucleotide.

15. The oligonucleotide of claim 14 wherein the ribonucleotide is a 2'-O-methylated ribonucleotide.

16. The oligonucleotide of claim 11 further comprising at least one ribonucleotide.

17. The oligonucleotide of claim 16 comprising at least one 3'-terminal 2'-O-methylated ribonucleotide.

18. The oligonucleotide of claim 16 comprising at least one 5'-terminal 2'-O-methylated ribonucleotide.

19. The oligonucleotide of claim 17 further comprising at least one 5'-terminal 2'-O-methylated ribonucleotide.

19. The oligonucleotide of claim 17 comprising at least two 5'-terminal 2'-O-methylated ribonucleotides and at least two 3'-terminal 2'-O-methylated ribonucleotides.

20. The oligonucleotide of claim 19 having modified internucleotide linkages.

21. The oligonucleotide of claim 20 having phosphorothioate internucleotide linkages.

22. The oligonucleotide of claim 1 which is capable of inhibiting CDK4 protein expression.

23. A method of regulating the G1 to S phase transition in a cell, comprising the step of administering to the cell an oligonucleotide of claim 1 in an amount sufficient to inhibit the transition.

24. The method of inhibiting the growth of a cancerous cell which has lost its G1 to S restriction point control, comprising the step of administering to the cell an oligonucleotide of claim 1 in an amount sufficient to inhibit transition of the cell from G1 to S.

25. A therapeutic composition comprising at least one oligonucleotide of claim 1 and a pharmaceutically acceptable carrier or diluent.

26. A method of treating a mammal afflicted with a tumor associated with the aberrant expression of CDK4, cyclin D1, or P16, the method comprising the step of administering to the mammal the therapeutic formulation of claim 25 in an amount sufficient to reduce tumor growth.

FIGURE 1

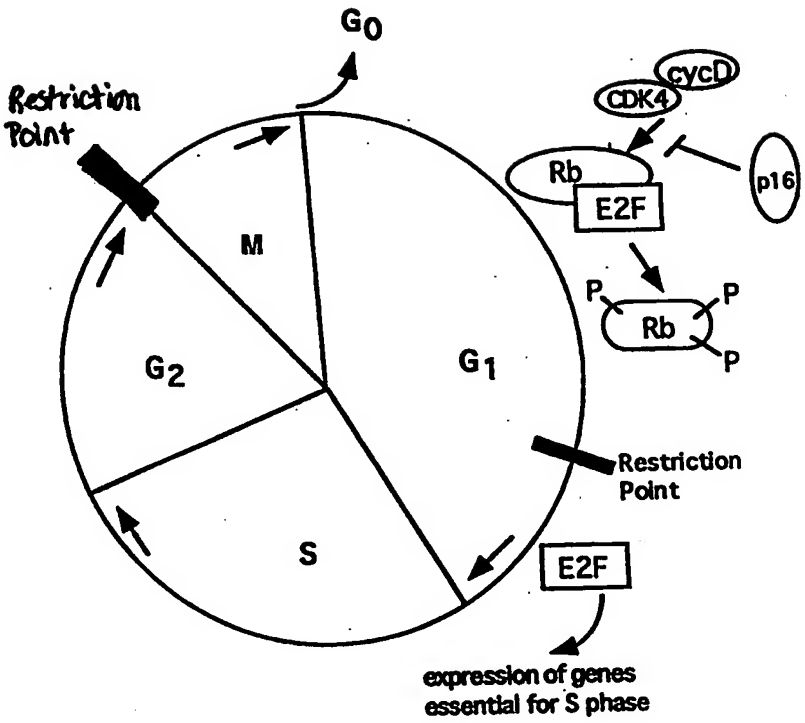

G0
Restriction Point
cycD
CDK4
Rb
E2F
p16
P
P
Rb
P
P
M
G2
G1
S
Restriction Point
E2F
expression of genes essential for S phase

FIGURE 2A

CDK4 Genomic Sequence

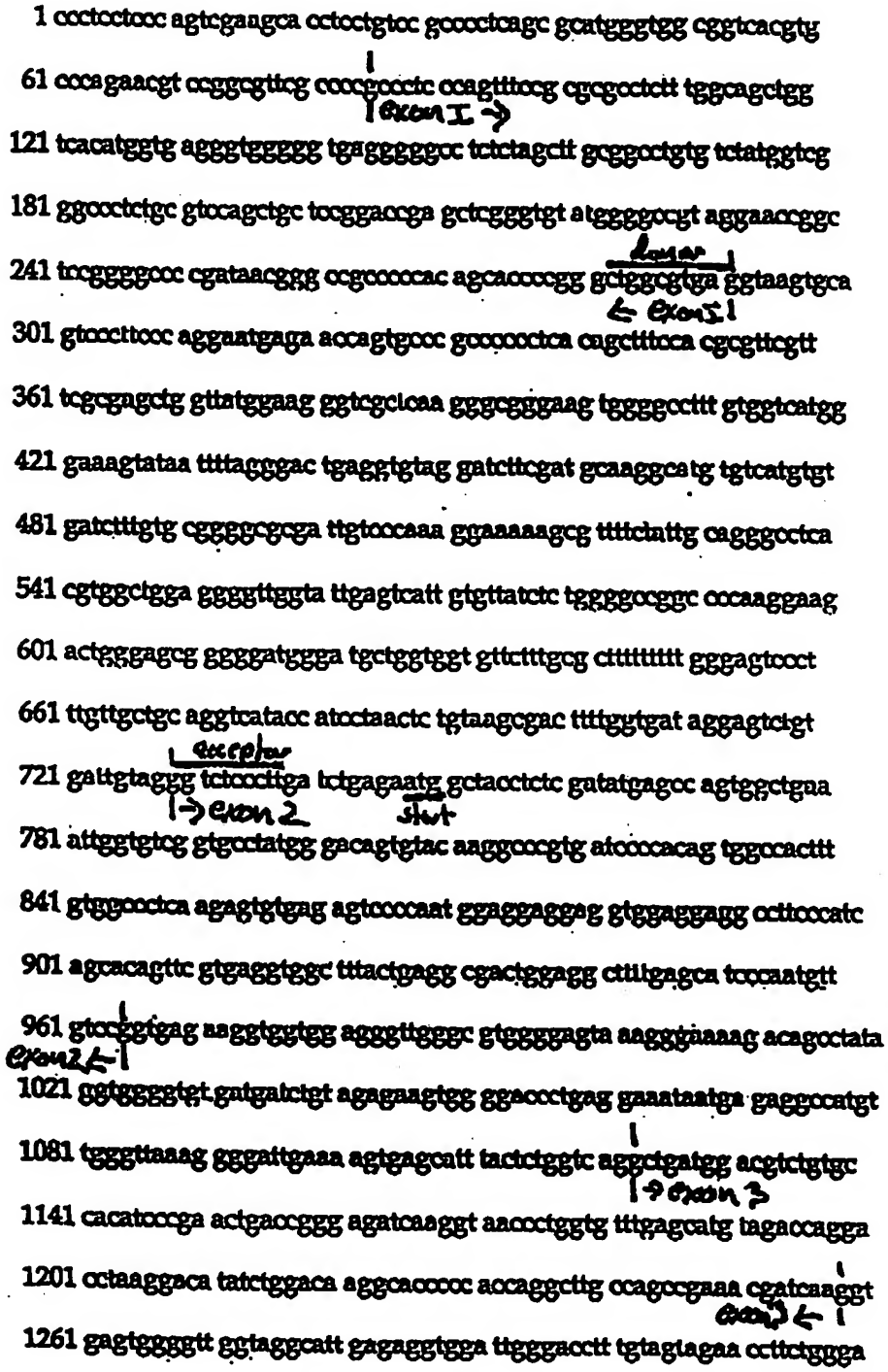


FIGURE 2A (Continued)

1321 tttcaggtat ggtgcctagt ttccagtgca tctgtacctc ccccttgaa actaggatct

1381 gatgccgccag tttctaagag gcctagattt ccttcatgcc aattgcatcg ttcaccgaga

1441 tctgaagcca gagaacattc tggtgacaag tggtggaaca gtcaagctgg ctgacttgg

1501 cctggccaga atctacagct accagatggc acttacaccc gtggtcagta gaaagatggt

1561 accaaaatgg gttctggttg ggaataggag agtgattgcc cgtagcaatt gagaagtcat

1621 gtgcttcatg tgttcagtca agcaagttgt gtttcatggt aacccatggg gtcccatcc

1681 attcttccta ttccctttag gttgttacac tctggtaccg agctcccgaa gttcttctgc

1741 agtccacata tgcaacacct gtggacatgt ggagtgttgg ctgtatcttt gcagagatgt

1801 ttcgtcgaaa gtatgggacc cacataccct gyactaccti gaattcccca aatcgcttgt

1861 tcataaacca catccatacc ttgcccattc ttttttttg agaccagggc ttgctgtgtt

1921 gcccaggctg gattgcaatg gcatgatcac agctcactgc agcttcaacc tcctgggctc

1981 aagtgatcct cccatctcag cttcccaact agctgacact acaggcacgc acctccatgc

2041 ttggctagtt tgttaatatt tttatagaga tggggtctca gtatattgcc caggctggtc

2101 ttgaactctt gcactcaagc aatcctccca ccctacctc ccaaagtagc ataagctact

2161 gcatctggcc ccattctttt acttgcgtac tactaacttg cccatagcag aaagctctga

2221 aatgttctgg aattaggaac ttcatatccc tttattctct ttatttttta tttatttatt

2281 tatttattta tttatttatt gagataaggt ttcactctgn nacccaggct ggagtncagt

2341 ggcccaatta nagctcactg tancctctac ctcctgggct aaagmaatcc tcccatctca

2401 gcccctgag tanctgagac taaaggtgca cgccacccatg actggctttt ttttttttta

2461 gatggagtct tgctctgtcg ccaggctgga gtgcagtagt gcgatctctg ctcactgcaa

2521 cctccacctc ccagattcaa gcaattctct tgactcagcc tcccaagtag ctgggaccac

2581 aggtgcacgc caccatgctc agctaatttt tgtacttta gtaatgacag gtttcaccat

2641 gttggccagg atggtctcga tctcttgacc tcatgatcca cccacatcag actcccaaag

FIGURE 2A (Continued)

2701 tgctaggatt acaggcgtga gcnnnngcac ctggcatttc ttttttttta aaaaaagaga

2761 caaggtcttg cttgcccagg ctgatctaga actcctgggc tcaagcagtc ctctcacctc

2821 agcatcccaa agtgctggaa ttgttggcct ttattcccta tacttcctat ttgagccac

2881 taagcagtaa ccatttcaact aagatatctt tgaaaatgac tgctacctta tatcccttct

2941 cacctaggc ctctcttctg tggaaactct gaagccgacc agttgggcaa aatctttgag

3001 taagtgacca acatgggaga aaaagatttt ctatttctgag tcctctttct gctgaaccca

3061 ggatggcaac tggctctgcc atgggggatgg gaactggagg aocctcctga ccagagttct

3121 cctgtcccc acagcctgat tgggctgcct ccagaggatg actggcctcg agatgtatcc

3181 ctgccccgtg gagccttcc ccccagaggg ccccgcccag tgcagtcggt ggtacctgag

3241 atggaggagt cgggagcaca gctgctgctg gtaactggag atggctgtgg gcacagggaa

3301 agaaatagag actgggggaaa gaaatagagc agtatgcagg gcctggcca ctgtggttaa

3361 tgaaacttgg ttggtagatg gtctgtagtt ttattacag ctgcaaatag ccacccacag

3421 agaaggatat agaagagaac ccatcctggc tgggcacggt ggctcacgcc tgtaatccca

3481 gcactttggg aggccaaggt gggcgtatca cctgaggtca ggagttcgag accagcctgg

3541 ccaacatggt gaaacctcgt ctctactaaa agtacaaaaa taagccgggg gtggtggcac

3601 acgcctgtaa tctcagctac ttgggaggct gaggtaggag aatcacttca actcaggagg

3661 cggaggttgc agtgagctga gatcatacca ttggcactcc agcctgggtg atagagcgag

3721 actccgtctn caaaaaaaaa aaaaaagaaa aaagaagaaa gctcatccca ggtattgttg

3781 tgggtggcag aagctgtttt cttcatggtt ttctgacctt tgcctctccc ctcaggaaat

3841 gctgactttt aaccccacaca agcgaatctc tgccttccga gctctgcagc actcttatct

3901 acataaggat gaaggtaatc cggagtgagc aatggagtgg ctgccatgga aggaagaaaa

3961 gctgccattt ccctctgga cactgagagg gcaatctttg cctttatctc tgaggctatg

4021 gagggtcctc ctccatcttt ctacagagat tactttgctg ccttaatgac attcccctcc

FIGURE 2A (Continued)

4081 cacctctcct tttgaggctt ctcctictcc ttcccatttc tctacactaa ggggtatgtt

4141 ccctcttgtc cctttcccta ccttatatt tggggtcctt ttttatacag gaaaaacaaa

4201 accaaaagaa awaatggccc tttttttttt ttt

FIGURE 2B

CDK4 cDNA Sequence

1 agccctccca gttccgcgc gcgtctttgg cagctggtca catggtgagg gtgggggtga

61 gggggcctct ctagcttgcg gcctgtgtct atggtcgggc cctctgcgtc cagctgctcc

121 ggaccgagct cgggtgtatg gggccgtagg aaccggctcc ggggccccga taacgggccg

181 ccccacagc accccgggct ggcgtga><ggg tctcccttga tctgagaatg gctacctctc

241 gatatgagcc agtggctgaa attggtgtcg gtgcctatgg gacagtgtac aaggcccgtg

301 atcccacag tggccacttt gtggccctca agagtgtgag agtccccaat ggaggaggag

361 gtggaggagg cctcccatc agcacagttc gtgaggtggc ttactgagg cgactggagg

421 cttttgagca tcccaatgtt gtccg><gctga tggacgtctg tgccacatcc cgaactgacc

481 gggagatcaa ggtaaccctg gtgtttgagc atgtagacca ggacctaagg acatatctgg

541 acaaggcacc cccaccaggc ttgccagccg aaacgatcaa><ggatctgatg cgccagtttc

601 taagaggcct agatttcctt catgccaatt gcatcgttca ccgagatctg aagccagaga

661 acatttctggt gacaagtggt ggaacagtca agctggctga ctttggcctg gccagaatct

721 acagctacca gatggcactt acacccgt><gg ttgttacact ctggtaccga gctcccgaag

781 ttcttctgca gtccacatat gcaacaactg tggacatgtg gagtgtggc tgtatctttg

841 cagagatgtt tcgtcgaaa><g cctctcttct gtggaaactc tgaagccgac cagttgggca

901 aaatctttga><cctgattggg ctgcctccag aggatgactg gcctcgagat gtatcccctgc

961 cccgtggagc ctttcccccc agagggcccc gcccagtgca gtcggtggta cctgagatgg

1021 aggagtcggg agcacagctg ctgctg><gaaa tgctgacttt taacccacac aagcgaatct

1081 ctgcctttcg agctctgcag cactcttatc tacataagga tgaaggtaat ccggagtgag

1141 caatggagtg gctgccatgg aaggaagaaa agctgccatt tccctctgg acactgagag

FIGURE 2B (Continued)

1201 ggcaatcttt gccttatct ctgaggctat ggagggtcct cctccatctt tctacagaga

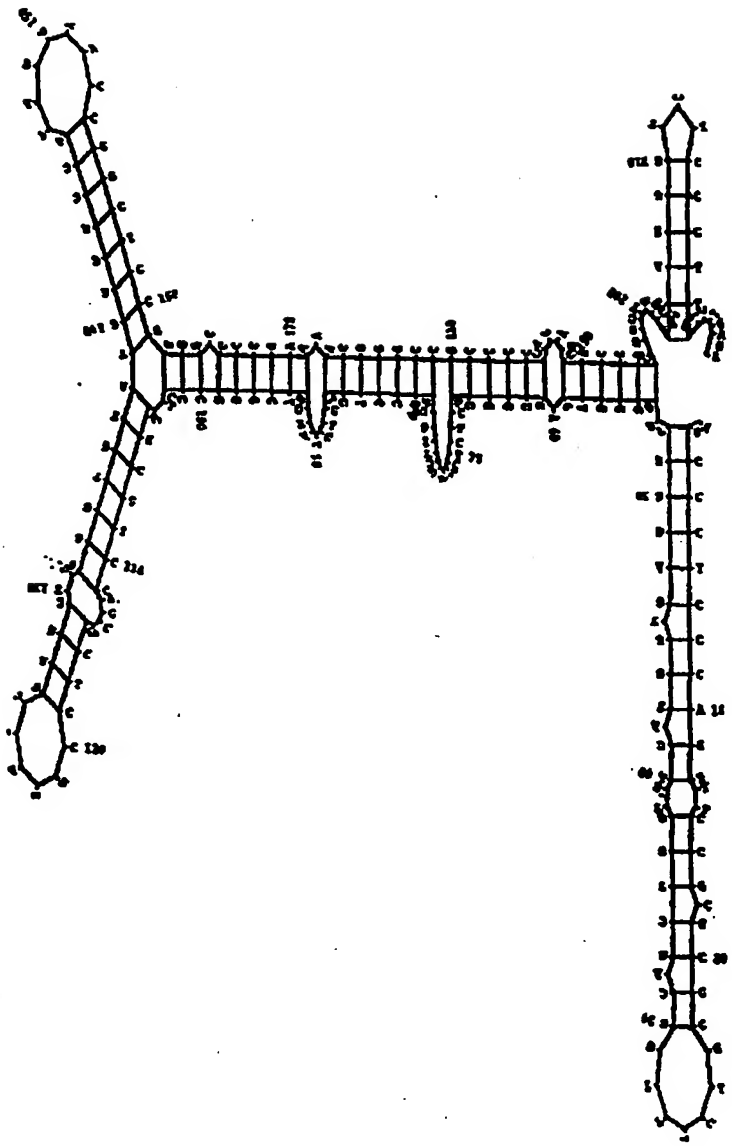
1261 ttacttigct gccttaatga catcccctc ccacctctcc ttttgaggct tctccttctc

1321 cttcccattt ctctacacta agggtatgt tccctcttgt ccctttccct accttatat

1381 ttgggtoct ttttataca ggaaaaacaa aac

FIGURE 3

CDK4 5'-UTR RNA Secondary Structure



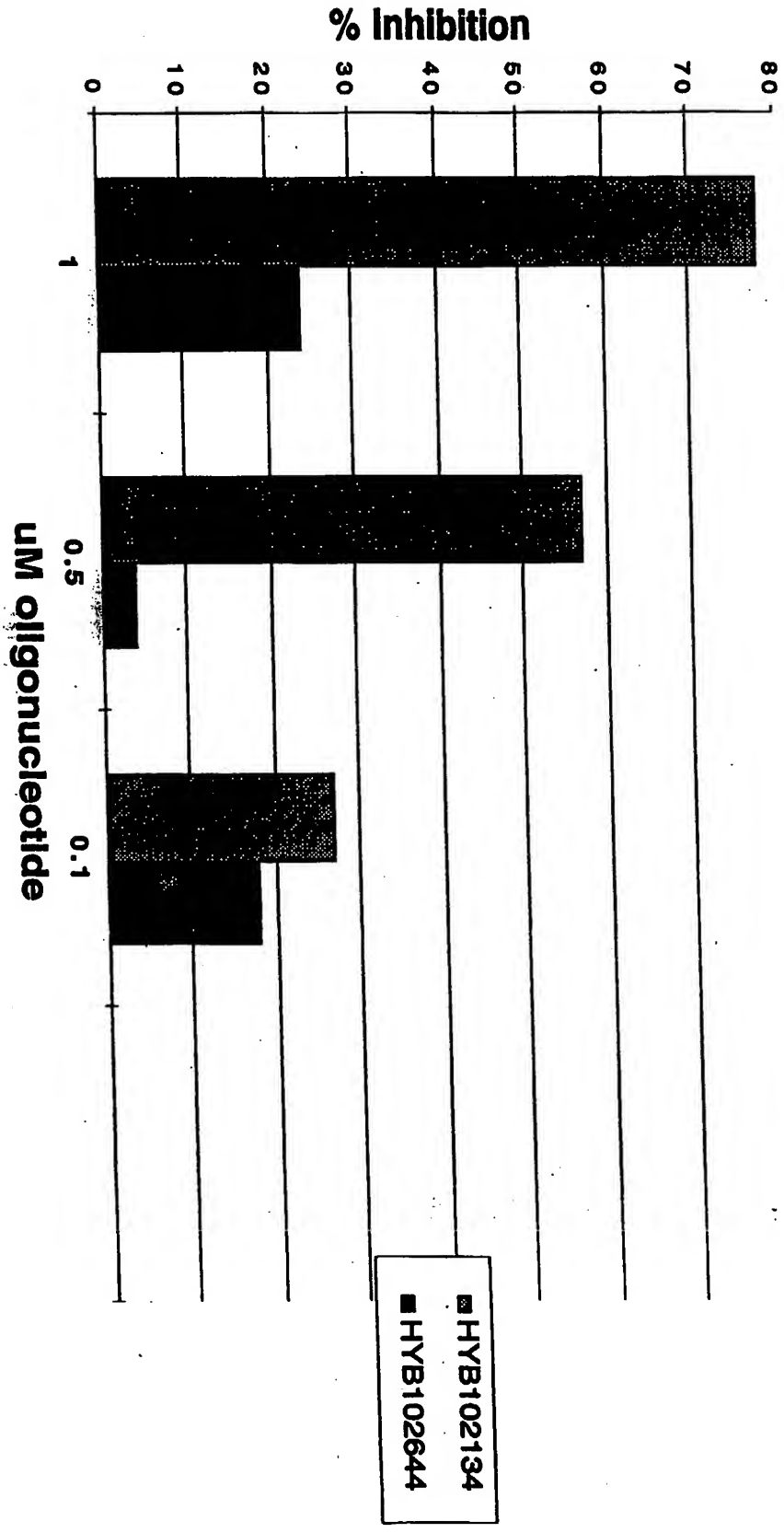

% Inhibition
0
10
20
30
40
50
60
70
80
1
0.5
0.1
uM oligonucleotide
HYB102134
HYB102644

FIG. 4

FIG. 5